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# Construction and characterization of an azurin analog for the purple copper site in cytochrome *c* oxidase

(blue copper/site-directed mutagenesis)

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**ABSTRACT** A protein analog of a purple copper center has been constructed from a recombinant blue copper protein (*Pseudomonas aeruginosa* azurin) by replacing the loop containing the three ligands to the blue copper center with the corresponding loop of the Cu<sub>A</sub> center in cytochrome *c* oxidase (COX) from *Paracoccus denitrificans*. The electronic absorption in the UV and visible region (UV-vis) and electron paramagnetic resonance (EPR) spectra of this analog are remarkably similar to those of the native Cu<sub>A</sub> center in COX from *Paracoccus denitrificans*. The above spectra can be obtained upon addition of a mixture of Cu<sup>2+</sup> and Cu<sup>+</sup>. Addition of Cu<sup>2+</sup> only results in a UV-vis spectrum consisting of absorptions from both a purple copper center and a blue copper center. This spectrum can be converted to the spectrum of a pure purple copper by a prolonged incubation in the air, or by addition of excess ascorbate. The azurin mutant reported here is an example of an engineered purple copper center with the  $A_{480}/A_{530}$  ratio greater than 1 and with no detectable hyperfines, similar to those of the Cu<sub>A</sub> sites in COX of bovine heart and of *Paracoccus denitrificans*.

Recently, there has been an increasing interest in the structure and function of purple copper proteins (1, 2). Examples of purple copper proteins are the Cu<sub>A</sub> centers in cytochrome *c* oxidase (COX) (3) and nitrous oxide reductase (N<sub>2</sub>OR) (4). Both copper centers share similar spectroscopic features, including the strong electronic absorption around 480 nm and 530 nm, and the characteristic resonance Raman Cu-S stretching frequency around 340 cm<sup>-1</sup> (5). Different structural models have been proposed based on spectroscopic studies and the conserved amino acid residues that appear to be involved in coordination of the copper: two cysteines, two histidines, and one methionine (2, 6–10). Many spectroscopic studies, including electron paramagnetic resonance (EPR) (7, 8) and extended x-ray absorption fine structure (9), and protein engineering studies (10) strongly suggest the existence of a mixed valence [Cu(1.5)··Cu(1.5)] binuclear center. This model has been confirmed by recent x-ray structural characterizations (11, 12).

Azurin is a blue copper protein whose structure has been well characterized by many spectroscopic techniques (13, 14) and x-ray crystallography (15). The copper-binding site consists of two histidines and one cysteine in a trigonal plane, with one methionine and one carbonyl oxygen weakly bound to the copper. Three of the ligands (His-117, Cys-112, and Met-121) fall into a loop between the two C-terminal  $\beta$ -strands (called the ligand loop hereafter, see Table 1). It has been shown by amino acid sequence homology (16) that the Greek key  $\beta$ -barrel fold of the azurin is a common structural motif in many blue copper proteins as well as in several copper centers in multicopper oxidases, including COX. The major difference

occurs in the amino acid sequence of the ligand loop (see Table 1). van der Oost and coworkers (10, 17) used this observation as the basis for engineering a blue copper center into the *Escherichia coli* cytochrome *o* quinol oxidase (CyoA). To create the blue copper center, the amino acid sequence of the loop that contains no potential ligands to the blue copper was replaced with the corresponding sequences for a blue copper protein amicyanin. A similar strategy was used to introduce a purple Cu<sub>A</sub> site into the CyoA (10, 17) and into amicyanin (18).

The ultimate goal of the research in this area is to gain an understanding of the detailed structure of this prevalent class of copper proteins and of how this structure determines their biophysical properties (electronic absorption and EPR spectra, for example) and redox behavior. The synthesis and study of structural and functional analogs of metalloproteins can provide an effective approach to achieve these goals (19). To this end, several small copper–thiolate complexes have been synthesized (20, 21, 39), but none has yet been created that mimics the spectroscopic features of the purple copper center in proteins. The introduction of the appropriate ligands into the relatively rigid skeletal framework of proteins can provide an alternative approach that has proved very powerful, as demonstrated by the success of engineering a purple Cu<sub>A</sub> site into CyoA (10, 17) and amicyanin (18). Azurin provides a particularly attractive protein for this purpose as it can be crystallized for structural determination by x-ray diffraction. Many studies have also elucidated the electronic structure (13, 22, 23) and the electron transfer behavior (24, 25) of the blue copper site in azurin. With a Cu<sub>A</sub> analog of azurin, we should be able to study the behavior of blue and purple copper centers in the context of the same protein framework and thereby gain a deeper understanding of copper–thiolate centers in proteins.

We report herein the creation and characterization of an azurin mutant that contains the essential features of the loop sequence of the Cu<sub>A</sub> site of COX from *Paracoccus denitrificans*. This mutant accepts copper to give a protein whose electronic absorption in the UV and visible region (UV-vis) and EPR spectra are remarkably similar to those of the native Cu<sub>A</sub> center in COX of *Paracoccus denitrificans*. While the purple copper centers in both COX and N<sub>2</sub>OR share many similar spectroscopic features, they are different in the extent of the EPR hyperfine splittings in the *z* direction, with the copper center in N<sub>2</sub>OR having a clear seven-line hyperfine and the copper center in COX has no or limited hyperfine splittings. We notice that this distinction between the two proteins may not be as definitive as it is generally believed, since at least one N<sub>2</sub>OR has a partial hyperfine splitting (26). Instead, we note that the character of the EPR hyperfine splittings along the *z* axis may correlate with the relative intensities of absorp-

Table 1. Amino acid and DNA sequences of the ligand loop

Ligand loop	Sequence
Mutant 1	Cys Ser Glu Leu Cys Gly Ile Asn His . . . Ala Tyr Met TGC TCC GAA CTG TGC GGT ATC AAC CAC . . . GCT TAC ATG
Mutant 2	Cys Ser Glu Leu Cys Gly Ile Asn His . . . Ala Leu Met TGC TCC GAA CTG TGC GGT ATC AAC CAC . . . GCT CTG ATG
Azurin WT	Cys Thr Phe Pro . . . Gly . . . His Ser Ala Leu Met TGC ACT TTC CCG . . . GGT . . . CAC TCC GCA CTG ATG

WT, wild type.

tion bands at 480 nm and 530 nm; properties of the mutant we have prepared lie near one of the extremes of this correlation and are similar to those of the native Cu<sub>A</sub> site in bovine heart COX.

## MATERIALS AND METHODS

The mutant DNAs were constructed on a recombinant azurin gene from *Pseudomonas aeruginosa* (27) by using a PCR mutagenesis method (28). The DNA sequences were verified by the dideoxynucleotide method using an Applied Biosystem model 373 automated sequencer in the Biotechnology Center at the University of Illinois. The expression and purification of the mutant proteins were carried out as described (27, 29) with minor modifications. Briefly, after acidification of the protein extract with 0.1 vol of 0.5 M sodium acetate (pH 4.1), the clear supernatant was purified on a SP-Sepharose FF column (Pharmacia) by a GradiFrac system (Pharmacia) through a gradient of pH 4.1–5.8 in 50 mM ammonium acetate buffer. Apoazurin was eluted at around pH 5.2. Apoazurin was titrated with a 10 mM Cu<sup>+</sup>/Cu<sup>2+</sup> solution to saturation. The holoprotein was subjected to another purification on an SP Poros column (PerSeptive Biosystems, Cambridge, MA) by a BioCad/Sprint chromatography system (PerSeptive Biosystems). The resulting protein is more than 95% pure, as judged by SDS/PAGE.

The UV–vis characterization was carried out on a Cary 3 system (Varian) and the X-band EPR spectra were collected on a Bruker ESP 300. Purified CuCl was provided by Hong-Chang Liang and Patricia Shapley (University of Illinois, Urbana–Champaign). It was dissolved in degassed water under the flow of argon. Upon storage, some of the Cu<sup>+</sup> was oxidized to Cu<sup>2+</sup> to give a mixture of Cu<sup>+</sup>/Cu<sup>2+</sup>. The concentration of Cu<sup>2+</sup> in the mixture was determined by UV–vis absorption at 833 nm with an extinction coefficient of 11 M<sup>−1</sup>·cm<sup>−1</sup>. The extinction coefficients were estimated from the absorption of the protein solution after addition of known amount of Cu<sup>2+</sup> ions and before the metal-binding sites are saturated.

## RESULTS AND DISCUSSION

The amino acid sequences of the ligand loop of the two mutant proteins are shown in Table 1. The mutant 1 has the same sequence as the native Cu<sub>A</sub> from *Paracoccus denitrificans* (30). Addition of Cu<sup>2+</sup> to this mutant protein resulted in no strong absorption in the visible region (data not shown), suggesting that introduction of the new ligand loop sequence of Cu<sub>A</sub> site into azurin may cause certain steric conflicts between the new amino acids and other residues in this region. Replacing Tyr-120 with Leu makes the mutant 2 more closely resemble native azurin, while maintaining the potential ligands to Cu<sub>A</sub> center.

Addition of a mixture of Cu<sup>2+</sup> and Cu<sup>+</sup> to the colorless apoprotein of mutant 2 resulted in an intense purple solution. The UV–vis spectrum of mutant 2 shows absorption around 350, 485, 530, and 765 nm (Fig. 1). It is strikingly similar to that of the native Cu<sub>A</sub> in COX from *Paracoccus denitrificans* (31) (Fig. 1 *Inset*). The spectrum also appears similar to the spectra of Cu<sub>A</sub> centers from other organisms (10, 17, 18, 32) and the

copper center in N<sub>2</sub>OR (26, 33) (Table 2). The blue shift of the 765-nm band relative to that of the Cu<sub>A</sub> center in COX from *Paracoccus denitrificans* (808 nm) has been observed in other purple copper systems. For example, the corresponding band for the engineered Cu<sub>A</sub> in CytoA is located around 765 nm (10, 17). The position of this particular peak is also pH-dependent: it shows blue-shifts with decreasing pH (31). The estimated extinction coefficients of the absorption bands ( $\epsilon_{350} = 380$ ,  $\epsilon_{485} = 1550$ ,  $\epsilon_{530} = 1380$ , and  $\epsilon_{765} = 770$  M<sup>−1</sup>·cm<sup>−1</sup>) are also similar to those of other purple copper centers (see references in Table 2).

Addition of Cu<sup>2+</sup> to the colorless apoprotein of the mutant 2 resulted in a solution with a strong blue color. The spectrum consists of absorption bands around 350, 485, 530, 600, and 765 nm (data not shown). The 600-nm band decreases upon incubation at room temperature. The intensity of the 485-nm band increases only slightly during the process. Addition of an excess amount of ascorbate resulted in the immediate disappearance of the 600-nm band, with little noticeable effects on the position and intensity of the other absorption bands. The spectrum shown in Fig. 1 can be obtained with prolonged incubation (>12 h) at room temperature, addition of excess ascorbate, or a combination of both. The above observation indicates that addition of Cu<sup>2+</sup> ions to mutant 2 can result in a blue copper protein with the 600-nm band or a purple copper protein with 350-, 485-, 530-, and 765-nm bands. This is not surprising since the ligand loop introduced for the formation of the purple copper site also contains all the ligands for the formation of a blue copper site (see Table 1). Since only the

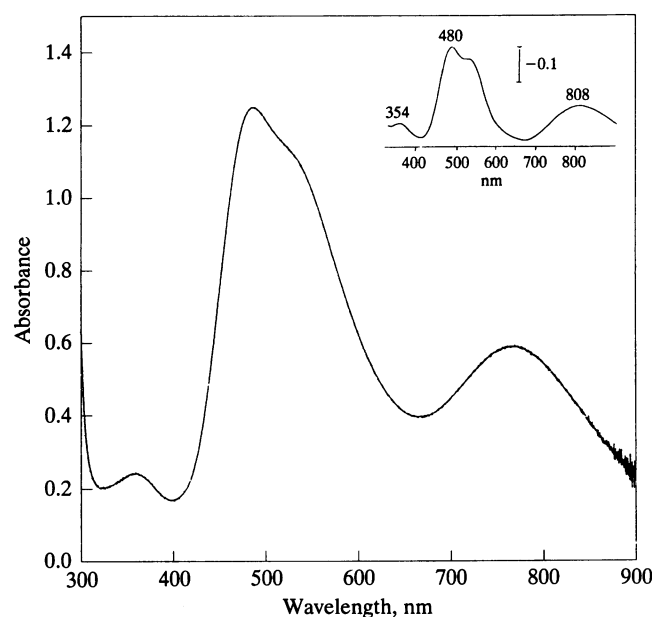


Fig. 1. Electronic absorption spectrum of azurin mutant 2 in 100 mM ammonium acetate (pH 5.2) at room temperature. (*Inset*) Spectrum of the soluble Cu<sub>A</sub> of COX from *Paracoccus denitrificans* at pH 6.0 (adapted from ref. 31).

Table 2. Electronic absorption data for selected purple copper proteins

Protein	Source	Absorption bands, nm	$A_{480}/A_{530}$	EPR hyperfines	Ref(s).
Cu <sub>A</sub> in COX	<i>Paracoccus denitrificans</i>	354, 480, 530, 808	1.15	No	31
Azurin mutant 2	<i>Pseudomonas aeruginosa</i>	350, 485, 530, 765	1.13	No	This work
Cu <sub>A</sub> in CAA <sub>3</sub>	<i>Bacillus subtilis</i>	365, 480, 530, 790	0.94	Partial	32
Amicyanin mutant	<i>Thiobacillus versutus</i>	360, 483, 532, 790	0.94	Partial	18
N <sub>2</sub> OR	<i>Achromobacter cycloclastes</i>	350, 481, 534, 780	0.98	Partial	26
Cu <sub>A</sub> in CyoA	<i>Escherichia coli</i>	358, 475, 536, 765	0.75	Partial*	10, 17
N <sub>2</sub> OR	<i>Pseudomonas stutzeri</i>	350, 480, 540, 780	0.75	Complete	33

\*The presence of another copper species makes it difficult to observe detail structure of hyperfines, even after careful spectral subtraction (17).

600-nm band disappears upon addition of ascorbate, the blue copper center of mutant 2 may have a higher redox potential than the purple copper center counterpart. The blue copper center is not stable to air, probably because of the tendency for disulfide bond formation between the cysteine in the blue copper site and the second free cysteine in the presence of Cu<sup>2+</sup>. This disulfide bond formation process can also provide the Cu<sup>+</sup> necessary for the formation of the mixed valent purple copper site.

The X-band EPR spectrum of the mutant 2 is shown in Fig. 2. Its similarity to the spectrum of the Cu<sub>A</sub> center in COX from *Paracoccus denitrificans* (31) is evident by both the shape and the parameters (mutant 2,  $g_z = 2.18$  and  $g_{x,y} = 2.06$ ; *Paracoccus denitrificans* Cu<sub>A</sub>,  $g_z = 2.20$  and  $g_{x,y} = 2.02$ – $2.04$ ).

The Cu<sub>A</sub> site has been engineered into two protein systems, one in CyoA (10, 17) and another in amicyanin (18). The UV–vis and EPR spectra of our mutant are similar to the engineered Cu<sub>A</sub> in both earlier systems in terms of the positions of the UV–vis absorptions and  $g$  values. However, the relative intensity of UV–vis absorptions and the EPR hyperfines along the  $z$  direction differ significantly. The ratio between the absorption around 480 nm and the absorption around 530 nm is higher than those of the Cu<sub>A</sub> sites introduced into CyoA and amicyanin. Moreover, no hyperfine splittings in the  $z$  direction are observed in our mutant, whereas the hyperfines partially resolved in the Cu<sub>A</sub> sites created in both CyoA and amicyanin.

An examination of published UV–vis and X-band EPR spectra of purple copper centers reveal a general correlation

between the  $A_{480}/A_{530}$  ratio and the extent of the display of hyperfines along the  $z$  direction (see Table 2). The Cu<sub>A</sub> centers both in our mutant and in COX from *Paracoccus denitrificans* have a ratio greater than 1 and no detectable hyperfines. Whereas the N<sub>2</sub>OR from *Pseudomonas stutzeri* has a ratio much lower than 1 and a clear seven-line hyperfine, the N<sub>2</sub>OR from *Achromobacter cycloclastes* has a ratio slightly lower than 1 and a partially resolvable hyperfines. The two previously engineered Cu<sub>A</sub> centers are in the same category as the N<sub>2</sub>OR from *Achromobacter cycloclastes*. A correlation of UV–vis absorption and EPR rhombicity has been found in blue copper proteins (34–36).

It has been shown that the Cu<sub>A</sub> center in COX shares many similar spectroscopic properties with the binuclear center in N<sub>2</sub>OR (5, 7). However, while N<sub>2</sub>OR shows a clear seven-line X-band EPR hyperfines along the  $z$  direction, characteristic of a mixed valent [Cu(1.5)·Cu(1.5)] binuclear center, the corresponding X-band EPR of Cu<sub>A</sub> center in bovine heart COX shows no hyperfines. Explaining this difference has been one of the main focuses of research in this field. Now we have shown that those purple copper centers with no hyperfines may also have the  $A_{480}/A_{530}$  ratio greater than 1. The mutant reported here is an example of an engineered purple copper site with no observable hyperfines and the  $A_{480}/A_{530}$  ratio greater than 1, characteristics that are strikingly similar to those of the native Cu<sub>A</sub> sites in COX of bovine heart.

The electronic structure and spectrum of several models of the binuclear metal site in the soluble Cu<sub>A</sub> domain of COX have been calculated (37). The models were constructed based on the fusion of two blue copper sites. The strong sulfur-to-Cu(II) charge transfer band of the monomer was shown to split into two because of a dipole–dipole interaction between the two sites. In the same paper (37), the authors also pointed out that the spectrum is sensitive to the distance between the two S atoms of the cysteine residues. The influence of the Cu–Cu distance on the spectrum has also been discussed.

Blue copper centers show a correlation between UV–vis absorption and EPR rhombicity, and an explanation has been put forward on the basis of an electronic structural calculation (34–36). The structural origin of the correlation has been established and a possible functional consequence as the result of the fine tuning of the structure of the mononuclear blue copper site has also been suggested (34–36). The results confirm that it is possible to fine tune the properties of the metal-binding sites by rack-induced bonding (38). The development of rack-induced bonding concept and the confirmation of the fine tuning of the bonding have led to a comprehensive understanding of the spectroscopic properties of the mononuclear copper-thiolate centers and its correlation with the fine details of the structural and functional properties (23, 34–38). Further studies on purple copper centers such as the mutant presented here in the context of other engineered Cu<sub>A</sub> sites should also shed light on structural and functional properties of purple copper centers. An important characteristic that emerges from these detailed structural and functional

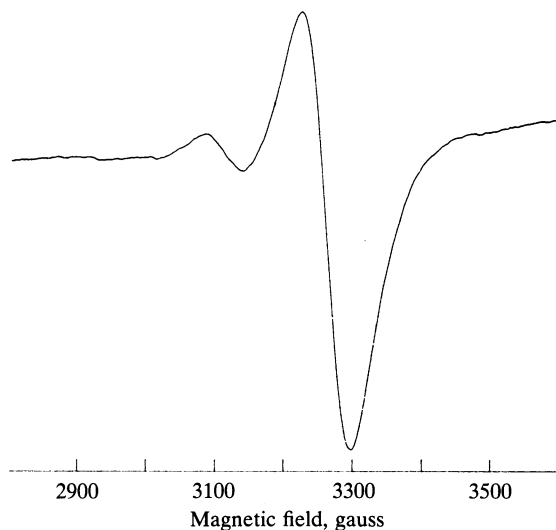


FIG. 2. X-band EPR spectrum of azurin mutant 2 in 100 mM ammonium acetate (pH 5.2) at liquid helium temperature (4 K). Instrument settings: microwave frequency, 9.42 GHz; microwave power, 20 mW; modulation amplitude, 10 gauss; modulation frequency, 100 kHz.

studies is how aspects of protein structure, more subtle than those involving the identity of the ligands that form the metal ligand site, can profoundly influence the spectroscopic and functional properties of metalloproteins.

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